Universidade de Aveiro Departamento de Química
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Valor biomarcador de glicanos *O*-GalNAc no controlo terapêutico de NMIBC

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, especialização em Bioquímica Clínica, realizada sob a orientação científica do Doutor José Alexandre Ribeiro de Castro Ferreira do IPO-Porto e do Departamento de Química da Universidade de Aveiro e Professor Doutor Francisco Amado do Departamento de Química da Universidade de Aveiro

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Agradecimentos

Para a conclusão deste trabalho, contribuíram os conhecimentos adquiridos ao longo dos cinco anos de formação académica que antecederam a realização do mesmo. Deste modo, cumpro assim o dever de agradecer às seguintes pessoas, pelo apoio prestado:

- Ao Doutor José Alexandre Ferreira, meu orientador, pela disponibilidade inigualável, pelo rigor científico e metodológico e pelo incentivo constante com que me guiou ao longo deste caminho.

- Ao Professor Doutor Lúcio Lara Santos por me ter recebido no Grupo de Patologia Experimental e Terapêutica do IPO do Porto, por toda a orientação e por ter facilitado a obtenção de amostras e informações clínicas.

- Ao Professor Doutor Francisco Amado pela importante partilha de conhecimentos no início desta dissertação.

- Ao Mestre Luís Lima, por todo o apoio e colaboração, bem como, por toda a disponibilidade na partilha científica.

-Ás mestres: Sofia Pereira e Céu Costa bem como á Daniela Oliveira pelo conhecimento transmitido sobre imunohistoquímica.

- À Elisabete Fernandes, minha colega de laboratório e distinta amiga, pelo companheirismo, pelo carinho, pelas longas conversas de incentivo e por todos os memoráveis momentos passados.

- Aos meus colegas de curso: Inês, Nuno, Márcio, Hugo, Paulo, Duarte, Marta, Sofi, Ana Sofia, Mara, Lisandra e Tiago Camilo por terem tornado estes últimos dois anos inesquecíveis.

- Às insubstituíveis: Bianca, Vera, Armanda e Vanessa por me aturarem, acompanharem permanentemente, e por serem as minhas grandes amigas.

- Ao Sales, Susana e Élia porque sem vocês nunca seria a mesma coisa.

- Aos meus grandes amigos: Alexandre, Artur, Du, Mouro, Espanhol, Tiago F., André, João, C.Freitas e ao César pelas brincadeiras, carinho e amizade.

- Aos meus pais (Armando e Emília) por me proporcionarem esta oportunidade e por todo o apoio incondicional.

- À minha irmã (Cátia) por todas as conversas, aventuras e igualmente apoio incondicional.

- Aos meus avós (António, Fortunato e Maria) pelos bons conselhos e por todo o acompanhamento desde os meus primeiros dias de vida.

- Aos restantes membros da família, sem exceção, por todo o carinho, apoio e incentivo.

-E as todos aqueles que de alguma forma contribuíram para que esta caminhada chegasse ao seu destino. Muito Obrigada!

palavras-chave

Deficiência em LCHAD; oxidação mitocondrial de ácidos gordos; análise do proteoma mitocondrial; nanoLC-MS/MS

Resumo

A deficiência em 3-hidroxiacil-CoA desidrogenase de ácidos gordos de cadeia longa (LCHAD) é uma desordem da oxidação lipídica mitocondrial que apesar de rara está associada a um mau prognóstico devido às suas graves consequências clínicas. Apesar de a implementação dos programas de rastreio neonatal em alguns países desenvolvidos, incluindo Portugal, ter contribuído para uma melhor compreensão das doenças metabólicas e para a prevenção das suas consequências, os mecanismos fisiopatológicos subjacentes à LCHAD ainda são pouco compreendidos.

No sentido de contribuir para a elucidação destes mecanismos, avaliou-se a plasticidade mitocondrial em resposta à deficiência em LCHAD. Assim, foram isoladas mitocôndrias de culturas de fibroblastos obtidas a partir de biópsias de pele de doentes com deficiência em LCHAD e o seu proteoma foi caracterizado e comparado com amostras obtidas de indivíduos saudáveis. Recorrendo a nanoLC-MS/MS 729 proteínas distintas foram identificadas, a grande maioria pertencente aos seguintes *clusters* funcionais “metabolismo”, “transporte”, “transdução de sinal”, “processos de desenvolvimento e geração de percursores de metabolitos e energia”. Da análise dos resultados obtidos com marcação com iTRAQs identificaram-se 40 proteínas diferentemente expressas entre os dois doentes com défice de LCHAD e os controlos entre elas estão *chaperones,* protéases, proteínas associadas ao metabolismo e ainda proteínas associadas ao *stress* oxidativo.

Em geral, este estudo permitiu a obtenção de uma perspetiva global da plasticidade do proteoma mitocondrial perante a deficiência em LCHAD e evidenciou as vias moleculares envolvidas na sua patogénese.

keywords

Bladder cancer, *O*-GalNac glycans, bacillus Calmette-Guérin , immunohistochemistry, mass spectrometry.

abstract

Bladder cancer is one of the most common cancers in humans and its incidence has been increasing during the past years. Seventy percent of newly diagnosed bladder cancers are classified as non-muscle-invasive bladder cancer (NMIBC) and are often associated with high rates of recurrence that require lifelong surveillance. Bacillus Calmette-Guérin (BCG) immunotherapy is the treatment of choice of this disease. However, at the moment there are no biomarkers to predict BCG therapeutics outcome.

It is now recognized that malignant transformations are often accompanied by a dysregulation at the *O*-glycosylation level with an overexpression of short chair *O*-glycans. Still, little is known regarding their expression in bladder tumors. Taking into account these considerations, this work begins by characterizing (Tn, STn, T, ST, S6T and S3T) antigens in 29 bladder tumors with different clinicopathological natures, low grade (n=17) and high grade (n=7) NMIBC and MIBC (n=5) by immunohistochemistry. This study has demonstrated that sialylated species predominate over neutral antigens (Tn and T). S6T had broader expression (approximately 90% of the tumors) being prevalent among low grade cases. In contrast, S3T expression was more restricted (48%) and more associated with high-grade. It was also observed that STn is characteristic of high grade and invasive tumors (71 and 80% respectively) that currently constitute the primary concern in bladder cancer management. In this context, it was determined the predictive value of STn in BCG immunotherapy outcome in a retrospective series of 94 high-risk tumors. This showed that positivity for this antigen was associated with a better response to treatment ($p = 0.024$). Moreover it Highlighted the trend towards an increased survival. The incorporation of the antigen S6T, which has the STn domain, into this model increased the overall response ($p = 0.001$) and survival ($p = 0.001$). Thus, important information was collected that may guide future studies in the understanding of tumor behaviour, definition of high-risk populations and therapeutics.

Abbreviations

β4/3GalTs	β1,-4/3 Galactosyltranferases
β3/4GnTs	β1-3/4 *N*-acetylglucosaminyltransferases
BC	Bladder Cancer
BCG	Bacillus Calmette Guérin
C1GalT	Core 1 β1-3 Galactosyltransferase or T synthase
C2GnT	Core 2 β1-6 *N*-acetylglucosaminyltransferase
C3GnT	Core 3 β1-3 *N*-acetylglucosaminyltransferase
CIS	Carcinoma in situ
CMP-Neu5Ac	Cytosine monophosphate *N*-acetyl neuraminic acid
Cosmc	Core 1 β1-3galactosyltranferase-specific molecular chaperone
ER	Endoplasmic Reticulum
Gal	Galactose
GalNAc	*N*-acetyl-galactosamine
GlcNAc	*N*-acetyl-glucosamine
MIBC	Muscle invasive bladder cancer
MS	Mass spectrometry
Neu5Ac	*N*-acetyl-neuraminic acid
NMIBC	Non-muscle invasive bladder cancer
ppGalNAc-Ts	Polypeptide *N*-acetylgalactosaminyltransferases
PTM	Post-translational modification
Ser	Serine
ST	Sialyl-T antigen
STn	Sialyl-Tn antigen
ST3Gal	α 2-3 Sialyltransferases galactosamine
ST6GalNAc	α 2-6 Sialyltransferases *N*-acetylgalactosamine
Thr	Threonine
UCC	Urothelial carcinoma cell
UDP-Gal	Uridine diphosphate - galactosamine
UDP-GalNAc	Uridine diphosphate - *N*-acetylgalactosamine
UDP-GlcNAc	Uridine diphosphate - *N*-acetylglucosamine

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Chapter I

State of the art

1. General Introduction

Bladder cancer (BC) is one of the fifth most common human malignancy worldwide and its incidence has been increasing in recent years [1-3]. Moreover, it's the second most common urological tumor, particularly after the fifth decade of life and mostly in males [4-6].

The BC includes different histopathological entities, the majority being urothelial carcinoma. Urothelial cell carcinoma or UCC is a heterogeneous disease in respect of its natural and prognostic histology and is observed in more than 90% of tumors [4, 7]. At diagnosis, more than 75% of UCC cases are superficial non-muscle invasive cancers (NMIBC) [2, 8]. These include carcinomas limited to the mucosa, such as carcinoma *in situ* (CIS) and non-invasive papillary carcinoma (pTa), and tumors of the stage T1 extending from the urothelium and penetrating the basement membrane to the lamina propria [2]. Patients with NMIBC have a 5-years survival rate of between 88-98% [9]. In opposite, the 5-year survival rate for patients presenting muscle invasive disease (MIBC, pT2-pT4) is 46-63%, and falls to only 15% in those with locally advanced and metastatic disease (Figure 1) [3, 10-12]. According to current guidelines [2] these tumors are often divided into low grade tumors (mostly Ta) and high grade tumors (Ta and T1) that exhibit poorly differentiated cells.

Even though most cases are superficial NMIBC, BC presents the highest recurrence rate among solid tumors [12, 13]. Moreover, approximately 30-40% presents an elevated risk of progression to muscle invasive disease correlated with poor prognosis [5, 14]. Presently, the risk of relapse and progression is based exclusively with several clinical and histopathological factors (number and size of tumors, number of previous relapses, local staging (T) degree of differentiation and CIS presence) combined on a scoring system defined by the European Organization for Research and Treatment of Cancer (EORTC) [4, 12]. This group comprehends low grade tumors with persistent recurrence, multifocal disease, carcinoma in situ (CIS) and high-grade papillary tumors. Yet, due to the heterogeneous biological behaviour of tumors and the inter/intra-observer variability the accuracy of these models is as high as 70% [15, 16]. As such, during the last two decades, large number molecular markers have been associated with high-risk disease. However, and despite promising results, no single marker is able to predict with accuracy tumor

behavior [16]. It is now consensual that the integration of multiple biomarkers can improve the predictive value of this model; still such a panel remains to be established.

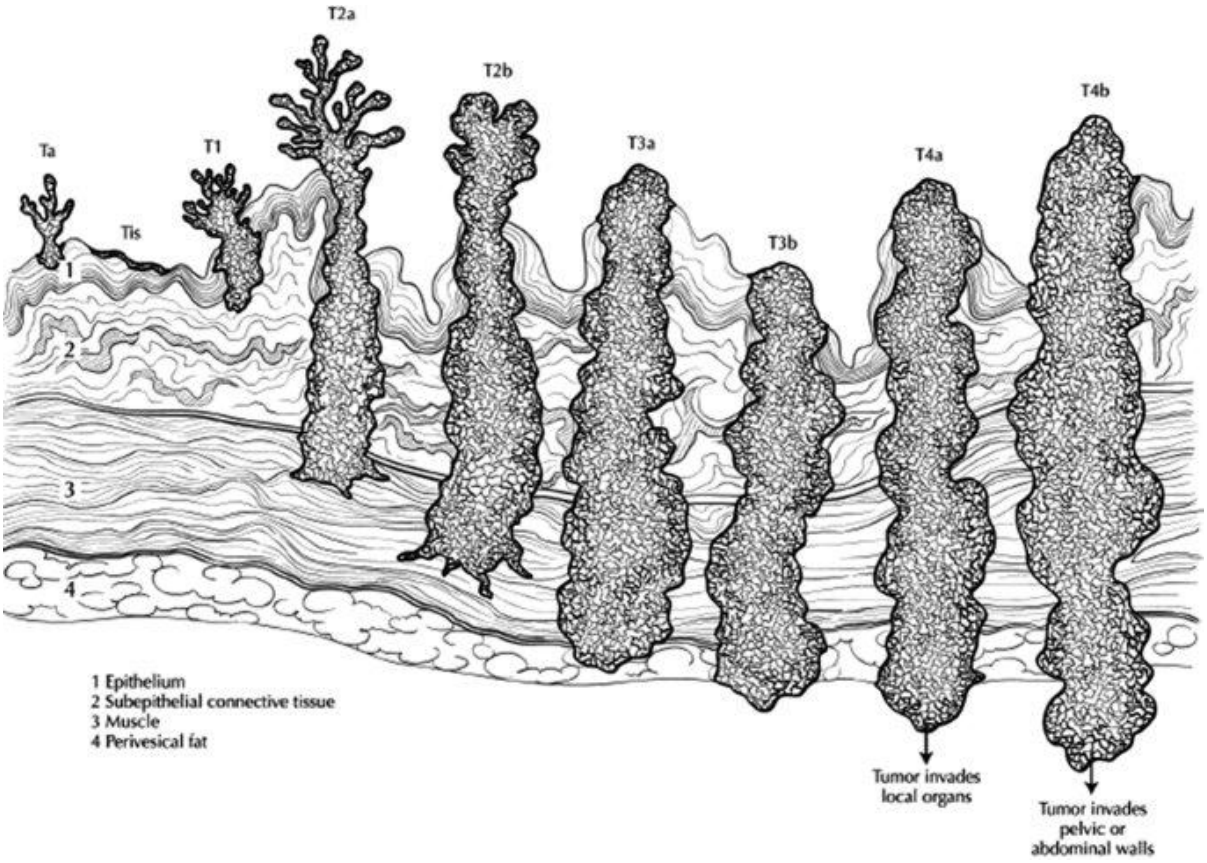

Figure 1: Illustration of the extent of tumor invasion in Bladder Cancer (Adapted from [3]).

Therapeutics based on the transurethral resection of the bladder tumor (TURBT) is usually enough for low-risk cases [17, 18]. However, to reduce recurrence rates and present disease progression, TURBT may be followed by an immediate postoperative instillation of chemotherapy, and/or an adjuvant course of intravesical chemotherapy or immunotherapy with Bacillus Calmette-Guérin; (BCG) [19-21]. Several studies have described that the BCG immunotherapy mechanism of action involves adhesion and internalization by cells, followed by tumor cell apoptosis and host adaptative immune response [10, 19, 20, 22]. Until now, intravesical BCG instillations have proven to be the most successful adjuvant treatment for patients with intermediate and high-risk NMIBC [2, 23, 24]. Still, 30–50% of patients fail to respond treatment and 15% have progression to muscle-invasive disease [13, 20]. Additionally, as there are no established markers to assist

prognostication, therapeutic failure is currently defined base either on recurrence or intolerance to BCG. The risk stratification is imperative for classifying patients with similar risks of recurrence and progression, and it helps to determine the appropriate management strategies for each risk category. Thus, the establishment of a biomarker panel to predict tumor behaviour, guiding the decision-making process between conservative treatment and radical approaches like cystectomy, is necessary. This is one of the most critical topics in high risk NMIBC management.

It is now recognized that, malignant transformation is often accompanied by a deregulation at the *O*-glycosylation level with an overexpression of short chair *O*-glycans. [25-27]. In particular, the expression of sialylated simple mucin type *O*-GalNAc glycans, have profound biological implications by influencing cell-cell interactions and cell-extracellular matrix, which consequently will interfere with tumor progression, metastasis, immune response, host-pathogen interaction and response to therapy [25]. These antigens are generally absent in healthy tissues and have been identified in different tumor types, including bladder cancers [28, 29]. Also, recent *in vitro* studies have demonstrated that cells over-expressing tumor-associated form STn presented higher levels of adhesion and internalization of the bacillus [97]. These observations are in agreement with other reports that BCG can bind human cell-surface glycans [19, 20]. According to all this information, alterations in *O*-glycosylation patterns, may determine the BCG therapeutics result, and so be explored as biomarkers. Yet, little is known about the pattern of expression of simple mucin type *O*-GalNAc glycans in bladder tumors.

2. *O*-Glycosylation in human cells

Glycosylation is one of the most ubiquitous and complex post-translational modification (PTM) and is estimated to occur on more than half of the proteins encoded in eukaryotic human genomes [31]. *O*-glycosylation is among the most common and is frequently associated with mucins due to the high density of putative glycosylation sites encountered in this class of proteins. However, *O*-glycans can be found in many other proteins. The *O*-glycosylation of proteins is generally initiated by the transfer of a *N*-acetyl-galactosamine (GalNAc) from UDP-GalNAc to hydroxyl groups in serine (Ser) or treonine (Thr) (Figure 2) [25, 32]. This is catalysed by a large family of proteins, termed polypeptide *N*-acetyl-galactosaminyltransferases GalNAc-transferases (ppGalNAc-Ts), in the Golgi complex, and results in the formation of the Tn antigen (GalNAcα-O-Ser/Thr) [33-36].

There are up to 20 different known isoforms of ppGalNAc-Ts which are differentially expressed according to the tissue [37, 38]. Moreover, they show different site-dependent specificities and act in a concerted and highly regulated manner, to determine the density and *O*-glycosylation of these sites [34]. After the formation of Tn antigen, increased levels of α2-6 sialyltransferases ST6GalNAc-I/II promote the sialylation of the respective epitope originating the sialyl-Tn antigen (Neu5Acα2-6GalNAcα-O-Ser/Thr) thus, prematurely ending chain extension. Yet, the elongation of the Tn antigen is the most common event in non-pathological conditions, [26, 27]. The second step towards elongation involves the transfer of galactose (Gal) Tn antigen, resulting in the T antigen or core 1 (Galβ1-3GalNAcα-O-Ser/Thr) formation [39]. This reaction is catalysed by core 1 β1-3N-galactosiltransferase (T sintase or C1GalT-1) [40]. On the other hand, depending on the tissue, the action of the core 3 β1-3N-acetylglucosaminiltransferase (C3GnT) on Tn antigen, may lead to the formation of core 3 (GlcNAcβ1-3GalNAcα-O-Ser/Thr) that is often a precursor of more complex structures. Namely, core 3 can be elongate by the core 2/4 β1N-acetilglucosaminiltransferase (C2GnT-2), resulting in the core 4 that can then result in oligomeric structures. However, the T antigen can also be sialylated by α2-3 ST3Gal-I/II/IV or α2-6 ST6GalNAc-II/III/IV stopping elongation [26]. ST3Gal-I,II and IV are known to promote the sialylation of the *O*-3 position of the Gal residue originating the S3T antigen (Neu5Acα2-3Galβ1-3GalNAcα-*O*-Ser/Thr). The α2-6 ST6GalNAc-II/III and IV promote the sialylation of the *O*-6 position of the GalNAc residue originating the STn-

like glycan, S6T (Galβ1-3(Neu5Acα2-6) GalNAcα-*O*-Ser/Thr). The concerted action of both enzymes originates the di-sialylated T antigen (DST) [25-27, 32]. Since the Tn and T antigens and their sialylated counterparts have been classically found in mucins they have been termed simple mucin-type *O*-GalNAc glycans. In addition, the mono-sialylated T antigens (S3T and S6T) are generally termed by some authors as sialyl T or cryptic T antigens (ST). Adding some confusion to this nomenclature, some authors using immunological approaches have also include the DST antigens as part of ST antigens. This resulted from the lack of monoclonal antibodies to each specific form of ST as ledding to their assessment using an anti-T antibody after treatment with a α-neuraminidase [41].

Still, the elongation of the T antigen normally prevails over this sialylated structures, by the action of the β1-6 N-acetilglucosaminiltransferase (C2GnT-1/3), originating the core 2 (GlcNAcβ1-6(Galβ1-3)GalNAcα-*O*-Ser/Thr). The elongation of core structures results from the action of N- β3/4acetilglucosaminiltransferase (β3/4 GnT) and β3/4N-acetilgalactosiltransferase (β3/4 GalT), leading to the formation of the oligosaccharide chains type 1 (Galβ1-3GlcNAc-R) and type 2 (Galβ1-4GlcNAc-R) [25, 32, 42]. These structures may be terminated by antigens Lewis (Le) and ABO blood group [43]. More specifically, the Lewis antigens results from modifications in the oligosaccharide chains, by addition of fucose and sialic acids. The Le^a, Le^b and sialyl-Le^a antigens derive from changes in the type 1 chain while, antigens Le^x, Le^y and sialyl-le^y antigens result from the addition of fucose residues and sialic acid in the type 2 chain [32]. Sialylation of Lewis determinants results from the action of α2-3 sialyltransferases, ST3Gal-III and ST3Gal-IV/VI. These enzymes promote the transfer of sialic acids to the *O*-3 position of Gal residues in type 1 and type 2 chains, resulting in the formation of the sLe^a and sLe^y antigens, respectively [32, 40].

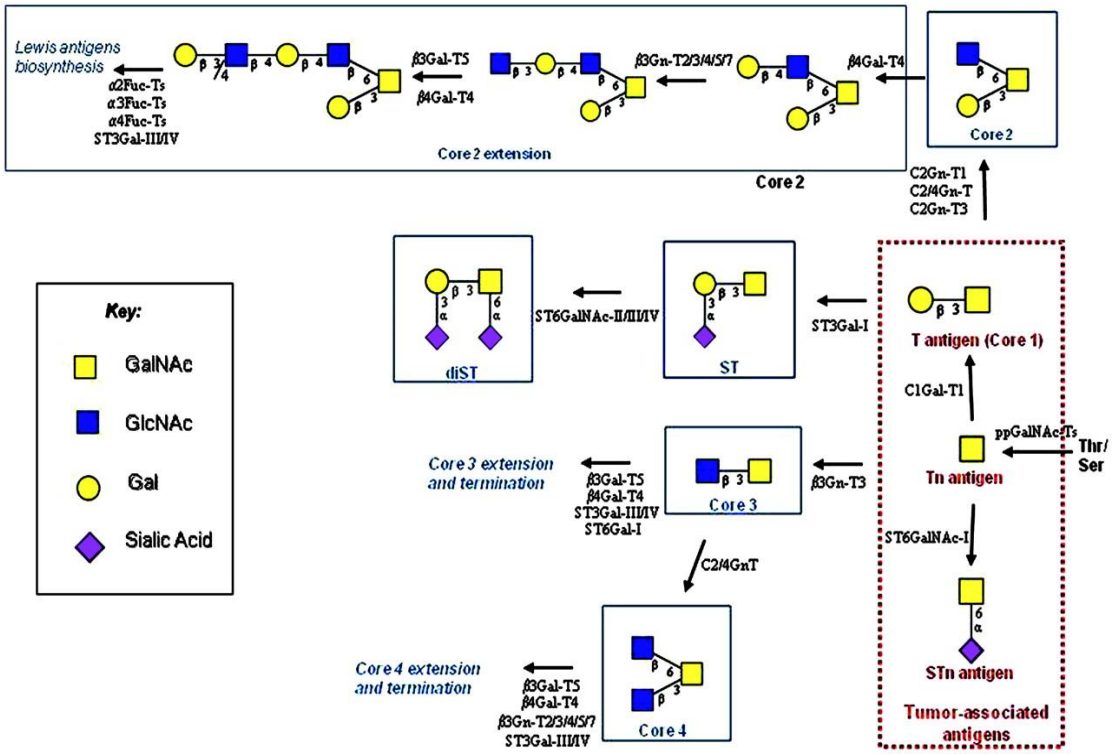

Figure 2: Representation of the pathways of biosynthesis of *O*-glycans of mucins common with the main tumor associated antigens highlighted: polypeptide *N*-acetylgalactosaminyltransferases (ppGalNAc-Ts); sialyltransferases ST6GalNAc and ST3/ST6Gal; core 1 β1-3 galactosyltransferase (C1GalT); core 3 β1-3 *N*-acetylglucosaminyltransferase (C3GnT); Core 2 β1-6-*N* acetylglucosaminyltransferase (C2GnT); fucosyltransferases (αFuc-Ts), β1-4/3 galactosyltranferases (β4/3Gal-Ts) and β1-3/4 *N*-acetylglucosaminyltransferases (β3/4Gn-Ts).(Adapted from [25]).

2.1. Alterations of *O*-glycosylation in tumors

The pattern of protein glycosylation is cell and tissue specific [36] and closely reflects the physiological status of the cell. Thereby, changes in glycan expression are being observed for several pathological conditions and in particular in during malignancy [27, 36, 44-48]. The first evidence of this fact resulted from the observation of plant lectins binding of tumor cells in animals [49]. In late 1980s, researchers concluded that, many of the available tumor specific antibodies showed reactivity against abnormal glycan structures [50, 51].

The most common changes occur in the *O*-glycosylation of tumor cell-surface and secreted glycoproteins; still the functional significance of these alterations is less well

understood [52]. These include the expression of abnormal core structures, increased synthesis of terminal structures, including Lewis blood group related antigens [53] have been reported. However, the majority of the studies consensually report a high expression of incomplete *O*-glycans such as sialyl Tn and other sialylated T-related antigens (Figure 3) [32]. The over-expression of these simple mucin type *O*-GalNAc glycans are common too many solid tumors, namely breast [45], oesophagus [54], colon [55], stomach [56], ovarian [57], bladder [28, 58, 59], among other tumors. Moreover, they have been associated with poor prognosis in patients with breast [45], colon and stomach [27] carcinoma. In particular, sTn is considered a pancarcinoma antigen, since it was found in several epithelial tumors, including colorectal [60], ovarian [61], breast [62], pancreatic [63] and gastric carcinomas [64], while absent in the healthy mucosa [65].

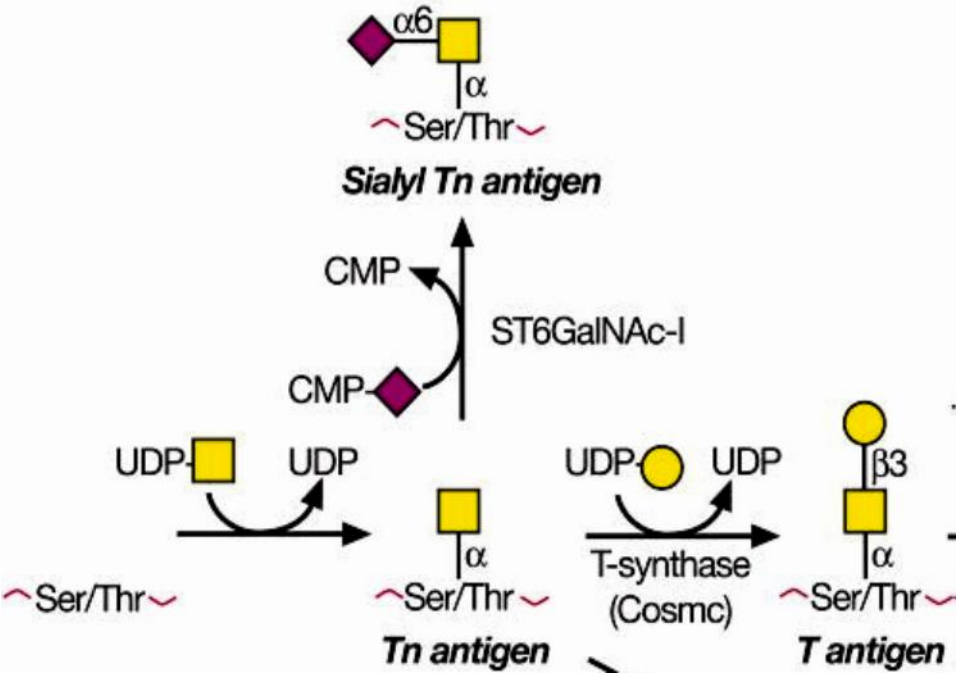

Figure 3: Structures of Thomsen-Friedenreich antigens (Adapted from [32])

The expression of T antigens was also found elevated in kidney, prostate, cervix, ovary, breast, liver, stomach and colorectal carcinomas [66]. The mono and disialylated forms of the T antigens can be found in healthy tissues but are significantly over-expressed in several tumors. These include testicular neoplasms [67], bladder tumors [28] and pancreatic adenocarcinomas [68]. However, very few studies distinguish the nature of the

type of sialylation present. Moreover, S6T has been mostly associated with malignancy *in vitro* [30, 69].

The majority of these alterations have been correlated with deregulations in the expression of glycosyltransferases [39, 70]. Specific changes have been described to occur in the initial steps of *O*-glycosylation. The enzymes responsible for initiating processing steps of *O*-glycans (GalNAc-Ts, and C1GalT C2GnT) are enriched in the cis-Golgi, while a later by the action of the sialyltransferases is effected in the trans-Golgi [70]. Recently, a new mode of regulation has been evidenced where the activation of protein kinase Src, selectively redistributes the enzymes located in the GC to the ER. Src kinase is a known tyrosine kinase proto-oncogene that is overexpressed in several human cancers [71]. Interestingly, the Src activity is not required for tumor growth, but is required for metastasis in several types of tumor [72]. These observations were correlated with the ability of citoplasmatic phosphorylation of the proto-oncogene, promoting cell adhesion. Recent findings suggest that, the activation of Src, promotes the relocation of GalNAc-T for RE resulting in an increase in the initiation locals of *O*-glycosylation, with of high levels of *O*-glycans short chain to the cell surface. In this context, it should be noted that the RE is a higher pH (around 7) for the GC (pH = 6.5) [73], making it possible that the catalytic activity and / or substrate preferences for the altered glycosyltransferases can also be a result of pH changing [36].

Other mechanisms leading to the accumulation of low molecular weight glycans include the upregulation/downregulation of glycosyltransferases, in particular sialyltransferases [74]. Namely, STn has been connoted with the high expression of sialyltransferases ST6GalNAc-I/II, but in particularly ST6GalNAc-I [30]. It has been advanced that this enzyme may compete with other glycosyltransferases, such as core 1 β1-3-Galactosyl-transferase [32] thus leading to a premature stop in *O*-glycosylation. Other mechanisms include the translocation of ST6GalNAC-I to early sub-compartments of the Golgi apparatus. The overexpression of STn may also result from a blockage in the expression of core 1 and core 3, making the Tn epitope available for sialyltransferases. Mutations in *Cosmc* gene that encodes Cosmc, a molecular chaperone required for the activity of core 1 β3-Gal-T has also been related with an enhanced synthesis of STn and Tn antigens [65, 75]. The high expression of STn is associated with aggressiveness and metastasis potential of the epithelial carcinomas [61] and is an independent indicator of

poor prognosis in gastric [76], ovarian [77] and colorectal cancer [60]. Naturally occurring antibodies to STn have been detected in gastrointestinal and ovarian cancer patients raising the possibility of immune response towards this epitope [78]. The ST has been associated in several studies with an increase in the expression of α2-3sialyltansferases, namely ST3Gal-I [28, 70]. Another study concerning pancreatic carcinoma has demonstrated that ST can result from a down regulation in core 2 β1-6-N-acetylglucosaminyltransferase, which is required for elongation of the mucin-type glycans [68]. The over-expression of UDP-Galactose transporters was also implicated in induction of expression of T antigen in tumor cells [32, 66]. Thus, several factors such as availability and location of the sugar donor and substrates, disruption of secretory organelles (CG and RE) as well as the presence of mutations, have been demonstrated to affect the activity and expression of glycosyltransfera a consequently the processing of *O*-glycans [79, 80].

2.1.1. Biological implication of simple mucin type *O*-GalNAc antigens

The structural alteration occurring in the *O*-glycosylation of proteins accompanying malignant transformations are reactively well identified. On the other hand, the biological alterations resulting from these events are still fairly understood. Yet, some studies have demonstrated a direct link between the aggressiveness of the cancer and the density of expression of simple mucin type *O*-GalNAc glycans [81-83]. In particular, *in vitro* studies have shown that the expression of STn contributes to alter cell recognition by the immune system [84], affects the adhesive properties of cancer cells, promotes invasion and metastasis [56, 64, 85]. Furthermore, STn appears to play a role in avoiding the elimination of metastatic cells in the blood stream by the immune system [65, 86]. This way, the sialic acids have repeatedly been implicated in the metastatic process. As a result, it is being explored as a serum biomarker of poor prognosis in gastric [76, 87], colorectal [88] and ovarian cancer carcinomas and in 74 tumor vaccines [77].

On the other hand, the T antigens are functionally important in cancer progression by allowing increased interaction or communication of the tumor cells with endogenous proteins. Namely, T antigens can bind circulating proteins, such as galectins, which in turn are involved in the regulation of cell progression, adhesion and metastasis [89]. These

interactions prevent the recognition of tumor cells by immune cells, thus providing a mechanism to escape the immune surveillance [66].

2.1.2. Alteration in expression of *O*-glycans in bladder cancer

The altered nature glycosylation of bladder cancer cell-surface and secreted glycoproteins has been long demonstrated [90-92]. It comprises aberrant *N*-glycan branching, over-sialylation, loss of ABO blood groups and/or abnormal Lewis (Le) antigen patterns [86]. Differentiated Le^y expression was identified for CIS and non-CIS [93] while the presence of SLe^x and/or SLe^a has been putatively correlated with invasive and metastatic potentials [86, 93]. However very few reports, published more than 20 years ago, have addressed the expression of simple mucin type *O*-GalNAc glycans [28, 41].

These studies have demonstrated that T and Tn are over-expressed in bladder carcinoma cells compared to normal cells where the expression is practically non-existent [27, 28]. In particular, Limas C. *et al*.,(1991) evaluated by immunohistochemistry the expression of the T antigen in biopsies from 56 patients at various stages of the disease. This author found that T was characteristic of more advanced stages and was almost absent from the healthy urothelium [29]. Also, the studies by Langkilde *et al.,(*1995) concluded that there is a correlation between T-antigen expression and recurrence or progression of initially non or superficially invasive human bladder tumors [94]. An additional study by Yamada *et al.*,(1988) [95] has demonstrated that cryptic T antigens (ST comprising S6T, S3T and DST) was normally expressed by non-invasive or superficially invasive (Ta or T1) papillary tumors. These authors added that ST expression was partially or completely lost in tumors that showed malignant progression after frequent recurrences. In line with these observations, recently Videira *et al*.,(2009) [28] demonstrated that ST3Gal-I plays the major role in the sialylation of the T antigen in bladder cancer. According to this study, the overexpression of ST3Gal-I seems to be part of the initial oncogenic transformation of bladder and can be considered when predicting cancer progression and recurrence [28]. At this point the expression pattern of STn as well as the individual expression of S3T and S6T remains unknown.

Chapter II

Aim and Scopes

1. Aim and Scopes

The management of high-risk bladder tumors poses a major challenge due to the high volume of recurrences and increased risk of progression to invasion. Currently the most efficient therapeutics includes transurothelial resection of the tumor followed by immunotherapy with BCG. However, this approach entails health risks and often fails to prevent poor outcome. Upon therapeutic failure and/or muscle invasion the patient is faced with cystectomy [2]. Hence, bladder cancer represents an opportunity and challenge for molecularly-targeted therapy, making of the molecular characterization of the tumors a mandatory task. Also, foreseeing those best served by an alternative or even more aggressive such as early cystectomy, would avoid disease progression, reduce disease burden and decrease health expenses. However, at the moment there are no biomarkers to predict BCG therapeutics outcome [20].

It is now well established that tumor cells express simple mucin type *O*-GalNAc glycans [27, 28] that contribute to malignancy and constitute a major source of cancer biomarkers [25]. Moreover they have been explored in cancer vaccines [78]. Still, little is known about the expression of these glycans by bladder tumors. Based on these considerations, the first part of the present work aims to characterize the expression of these antigens in BC. Considering their association with increase malignant potential [24], it is hypothesized high-risk cases may present distinct *O*-glycan patterns. The second part of the work is dedicated to determine the predictive value of altered *O*-glycosylation associated with high-risk tumors in the outcome of immunotherapy with BCG.

Chapter III

Analytical Approaches

1. Analytical Approaches

The identification of *O*-glycosylated epitopes (T, Tn, ST, STn, S6T and S3T) was performed in a prospective series of 29 bladder tumor tissues with different histological natures (low and high grade NMIBC and MIBC) from patients from the Portuguese Oncology Institute of Porto. This was done by immunohistochemistry in formalin fixed paraffin embedded (FFPE) using a combination of monoclonal antibodies and enzymatic treatments that have been resumed in Table 1. The observed structures where further validate by nanoLC-ESI-MS/MS. Briefly, prior to MS analysis, the proteins were extracted from the tissue and the *O*-glycans were isolated by reductive beta elimination followed by permethylation. This procedure equalized their chemical properties rending them hydrophobic and allowing their detection in positive ion mode [96]. Moreover, it stabilized labile residues such as sialic acids [96]. The second part of the work concerned the evaluation the O-glycans associated with high-risk tumors, by immunohistochemistry, in a retrospective series of 94 NMIBC cases treated with BCG collected by the Pathology and Experimental Therapeutics of the Portuguese Institute of Oncology, Porto).

Table 1: Studied antigens and monoclonal antibodies specific.

Antigen	Antibody	Dilution
T	3C9	1:10
ST	3C9 after neuraminidase	1:10
Tn	IE3	1:10
STn	TKH2	1:20
S6T	TKH2 after β (1,3) galactosidase	1:10
S3T	3C9 after α(2-3) Neuraminidase	1:10

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Chapter IV

Expression pattern of simple mucin type *O*-GalNAc glycans in Bladder Cancer

Abstract

Simple mucin type *O*-GalNAc glycans are commonly over-expressed in solid tumors and have therefore been explored as cancer biomarkers and in therapeutics. Still, little is known regarding their expression in bladder tumors. Taking this into consideration, the present work was dedicated to characterizing these antigens in 29 bladder tumors with different clinicopathological natures, low grade (n=17) and high grade (n=7) NMIBC and MIBC (n=5) by immunohistochemistry. The expression of the Tn, STn and T antigens were directly evaluated using monoclonal antibodies with 1E3, TKH2 and 3C9, respectively. The ST and S3T were determined using 3C9 after treatment with a α-neuraminidase and a α-(2-3)-neuraminidase, respectively. S6T was determined using TKH2 after treatment with a β-(1-3)-galactosidase.

This study has demonstrated that sialylated species predominate over neutral antigens Tn and T. S6T had broader expression (approximately 90% of the tumors) being prevalent among high grade cases. The S3T expression was more restricted (48%) being that, it was also associated with high grade. The presence of S6T and S3T was confirmed by mass spectrometry. To our knowledge, these antigens are being reported in bladder tumors for the first time. It was also observed that STn is characteristic of high grade and invasive tumors (71 and 80% respectively) that currently constitute the primary concern in bladder cancer management. Thus, important information was collected that may guide future studies in the understanding of tumor behaviour, definition of high-risk populations and therapeutics.

Keywords: Bladder cancer, *O*-GalNac glycans, sialylated antigens, immunohistochemistry, mass spectrometry.

1. Introduction

Bladder cancer is the fifth most common cancer in Western society and a growing concern in developing countries, as a result of demographic expansion and increased of life-expectancy [1, 2]. At presentation, most cases are superficial, non-muscle invasive bladder cancers (NMIBC), conservatively treated by complete transurethral resection (TUR) [3]. However, approximately half are burdened by a high volume of recurrences and present an elevated risk of progression to muscle invasive disease correlated with poor prognosis. According to the European Organization for Research and Treatment of Cancer (EORTC) this group mainly includes high grade papillary tumors and carcinoma in situ (CIS), both cases with multifocal lesions [3]. Thus, presently, the degree and stage are the most important prognostic factors for the risk of relapse and progression. Yet, due to the heterogeneous biological behaviour of tumors and the inter/intra-observer variability the accuracy of these models is as high as 70% [4, 5]. It is now consensual that the integration of multiple biomarkers can improve the predictive value of this model, making of the molecular characterization of bladder tumors a mandatory enterprise.

Glycosylation is a common post-translational modification of proteins with more than 50% of eukaryotic proteins thought to be glycosylated [6]. The pattern of protein glycosylation is cell and tissue specific [7] and closely reflects the physiological status of the cell. Thereby, changes in glycan expression have been described for several pathological conditions and in particular cancer [8]. The altered nature glycosylation of bladder cancer cell-surface and secreted glycoproteins has been long demonstrated [9-11]. Few reports also refer that some bladder tumors may express the low-molecular weight, simple mucin-type *O*-GalNAc antigens Tn and T antigens and their sialylated counterparts STn and ST [12, 13]. These epitopes result from a premature stop in protein *O*-glycosylation and have been described to affect the adhesive properties of cancer cells, promote their invasive, metastatic and angiogenic potential and alter recognition by immune cells [14, 15]. Still, the low number of cases, the high heterogeneity of the populations, and the multiplicity of objectives behind the studies presented so far prevented the establishment of a pattern of expression for these glycans. Among the most studied are the sialylated forms of T antigens, whose over-expression has been found in some types of cancers (colon, prostate, cervix, ovary, breast) and correlated with worse prognosis [13, 16]. Still, sialylated T antigens have been classically determined based on

the reactivity to Peanut Agglutinin (PNA) [17] and/or monoclonal antibodies [18] towards the T antigen after the removal of sialic acids with a neuraminidase. The lack of discrimination between S3T and S6T led to classify them under the generalist designation of cryptic T antigens. Thus, the present work is dedicated to the characterization of simple mucin-type *O*-GalNAc antigens in bladder tumors of different clinicopathological natures.

2. Material and Methods

2.1. Patient and sampling

Twenty-nine formalin-fixed, paraffin embedded tissues (FFEP) were prospectively collected from 27 male and 2 female patients, mean age of 70 (year range 45–89), who underwent bladder surgery in the Portuguese Institute for Oncology of Porto (IPO-Porto, Portugal), between July 2011 and May 2012. Based on urothelial carcinoma grading and staging criteria of the World Health Organization, three different groups were considered, low-grade (n=17) and high grade (n=7) non muscle-invasive papillary bladder cancers (NMIBC) and muscle-invasive (n=5) cancers (MIBC). All procedures were performed under the approval of institutions Ethics Committee of IPO-Porto and HSJ-Lisbon after informed patient's consent. All clinicopathological information was obtained from patient´s clinical records.

2.2. Immunohistochemistry for simple mucin type *O*-GalNAc glycans

Formalin-fixed paraffin embedded (FFPE) urothelium sections were screened for the glycans of interest by immunohistochemistry using the avidin/biotin peroxidase method. Briefly, 3 μm sections were deparaffinised and hydrated with xylene and graded ethanol series, and exposed to 3% hydrogen peroxide in methanol for 20 minutes, to reduce endogenous peroxidase activity.

The expression of the Tn, sialyl-Tn and T antigens was then directly evaluated using mouse monoclonal antibodies 1E3, TKH2 and 3C9, respectively. The expression of cryptic T antigens ST, comprehending ST3, S6T and DST was accessed after treating the sections with an non linkage-specific α-neuraminidase from *Clostridium perfringens* (Sigma-Aldrich). The S3T antigen was determined using 3C9 after treating the sections with an α(2→3)-neuraminidase from *Streptococcus pneumonia* (Sigma-Aldrich). The S6T antigen was determined using TKH2 after treating the sections with a recombinant β-(1-3)-

Galactosidase from *Xanthomonas campestris* (R&D systems). Prior to analysis, the sections were blocked with normal serum from Vectastain Elite ABC peroxidase kit (Vector Lab) for 30 minutes to avoid non-specific staining and then incubated with the enzymes, whenever the case, for 2 hours at 37ºC. The sections were then further incubated with specific antibodies at 37ºC for 30 min. Afterwards the sections were washed with PBS-T and incubated at room temperature for 30 minutes with the diluted biotinylated secondary antibody and then with Vectastain Elite ABC reagent (Vector Lab). After washing in PBS-T, the sections were incubated with 3,3-diaminobenzidine tetrahydrochloride (ImmPACT™ DAB, Vector Labs) for 5 minutes at room temperature to visualize antibody binding sites. Finally, they were counterstained with hematoxylin for 1 minute. Positive and negative control sections of intestinal metaplasia were tested in parallel. The negative control sections were performed by adding BSA (5% in PBS) devoid of any immunogen.

A semi-quantitative approach was established to score the immunohistochemical labeling based on the intensity of staining and the percentage of cells that stained positively. The sialyl-Tn labeling was be assessed double-blindly by two independent observers and validated by an experienced pathologist. Whenever there was a disagreement, the slides were reviewed, and consensus was reached.

2.3. Protein extraction from FFPE bladder tumors

The proteins were extracted from FFPE sections (3x10 μm) after deparaffinization of the tissue using Q-proteome FFPE Tissue Kit (Quiagen). The amount of protein in each fraction was determined using the RC-DC kit (BioRad) and the purity of RNA extracts determined based on the ration between A_{280}/A_{260}.

2.4. Identification of simple mucin type *O*-GalNAc glycans by mass spectrometry

2.4.1. Isolation of *O*-glycans

Proteins extracted from FFPE tissues were first de-*O*-glycosylated by reductive β-elimination upon incubation with 50 mM NaOH and 1M $NaBH_4$ at 45ºC for 16 hours. The reaction was stopped with glacial acetic acid until no fizzing was observed and the samples were subsequently filtered using 10-kDa molecular weight cutoff (MWCO Millipore). The filtrated containing low molecular weight peptides, *O*-glycans and borate salts was recovered and incubated several times with methanol containing 5% (*v/v*) acetic acid under a stream of nitrogen to remove borates as methyl esters.

2.4.2. Stabilization of sialic acids by permethylation

The *O*-glycans were then permethylated to stabilize labile sialic acids under ESI conditions adopting a modification of the method by Ciucanu and kerek [19]. Briefly, the native *O*-glycans were dissolved in 100 μL of DMSO, and powdered NaOH containing trace amounts of water was added to the reaction medium. The mixture was sonicated for 30 min and frozen prior to the addition of 10 μL of CH_3I and then incubated under mild stirring for 1h. The permethylated oligosaccharide were recovered from the reaction mixture by extraction with dicloromethane and extensively washed with acidified water (pH 2.0) to avoid base-induced hydrolysis of permethylated sialic acids. The samples were then de-salted using Dowex ion-exchange resin (Dowex 50W-X8) and dried.

2.4.3. Nano-HPLC- ESI-MS/MS

The permethylated samples were redissolved in 20% acetonitrile (ACN) aqueous solution containing 0.1% formic acid (phase A). Analysis of the samples was carried out by an Ultimate 3000 at a constant flow rate of 300 nL/min (Dionex) with a linear gradient of 5 to 50% B (95% ACN aqueous solution containing 0.1%formic acid) in 40 min using a

Pepmap100 (C18; 3 μm particle size, 0.75 μm internal diameter, 15 cm in length) connected to an LXQ-MS (Thermo) interfaced with an online nano-electrospray ion source. The electrospray voltage was used at 2.0 kV. The MS was operated in the data-dependent mode, selecting the 4 most intense parent ions for collision-induced dissociation in the LXQ trap per full MS scan. Normalized collision energy was 35.0%. Dynamic exclusion was set to initiate a 60 s duration for ions analyzed twice within a 15 s interval. The scan range was set from *m/z* 200 to 2000. Peak assignments in MS and MS/MS spectra were performed using the GlycoWorkBench platform [20].

3. Results and Discussion

The change in the *O*-glycosylation pattern of bladder tumors has been long demonstrated [12, 17, 21]. However, only one study by Videira *et al*., (2009) [13] as addressed this matter in the last twenty years. Moreover, there is little information regarding the expression pattern of simple mucin type *O*-GalNAc glycans in bladder cancer and their correlation with current clinicopathological classifications as defined by WHO guidelines. Adding to this, no attempts have been made to differentiate the sialylated forms of the T antigen. As such, this work is dedicated to the identification of novel *O*-GalNAc antigens and characterization of their expression pattern in bladder cancer.

Herein, 29 tissues FFPE bladder sections comprehending low (n=17) and high grade (n=7) NMIBC and MIBC (n=5) were screened by immunohistochemistry for simple mucin type *O*-glycans, using specific monoclonal antibodies. These included the Tn, STn, T antigens and its monosialylated forms S3T and S6T, which is regarded as a rare *O*-glycosylation, until now, mostly observed *in vitro* [22, 23]. The overall expression of sialylated T antigens (ST; cryptic T antigen), comprising S3T, S6T and DST was also determined. Noteworthy, S3T and S6T are being evaluated for the first time in bladder tumors. Simple mucin type *O*-GalNAc antigens can be found in the plasmatic membrane and, to some extent in the cytoplasm of tumor cells. Figure 1 is representative of some examples of the fact in question. These observations are consistent with previous reports associating these antigens with membrane glycoproteins and proteins in secretion granules within the cell [24].

3.1. Expression of Tn, T and ST antigens

In addition, Table 1 shows that the Tn and T exhibit the lowest levels of expression (<30% of the total cases). However, while the Tn antigen is characteristic of high grade tumors (57%) and absent in MIBC, the T antigen is more expressed among MIBC cases (40%). It was also observed that the T antigen (24% in low grade) predominates over Tn (12% in low grade) in low grade papillary tumors. This suggests that core 1 synthase (T-synthase) expression may be reduced in high grade tumors. Conversely, ST antigen was

detected in all tumors, highlighting a predominance of sialylated structures over neutral forms. Thus, an over-expression and/or increased activity of sialyltransferases may be expected. In line with these observations, it has been described that several sialyltransferases known to promote the sialylation of the T antigens are overexpressed in solid tumors [12, 13, 16]. This was also the case of bladder cancer [13]. Table 1 further highlight that simple mucin-type *O*-GalNAc glycans are fairly observed within the tumors, in the majority of the cases not exceeding 15% of the section. Additional studies should be carried out in order to identify the influence of the clones exhibiting altered *O*-glycans in tumor biology.

Table 1: Expression of simple mucin type *O*-GalNAC glycans in BC of different clinicopathological natures determined by immunohistochemistry.

		Tn	STn	T	ST	S6T	S3T
No. of cases							
Low Grade	**17**						
-		15 (88%)	13 (76%)	13 (76%)	0 (0%)	2 (12%)	10 (59%)
+		2 (12%)	4 (24%)	4 (24%)	5 (29 %)	11(65%)	6 (35%)
++					9 (53%)	4 (24%)	1 (6%)
+++					1 (6%)		
++++					2 (12%)		
Positive Cases (n.%)		**2 (12%)**	**4 (24%)**	**4 (24%)**	**17 (100%)**	**15 (89%)**	**7 (41%)**
High Grade	**7**						
-		3 (43%)	2 (29%)	5 (71 %)	0 (0%)	0 (0%)	2 (29%)
+		4 (57%)	4 (57%)	2 (29 %)	2 (29%)	3 (43%)	4 (57%)
++			1 (14%)			3 (43%)	1 (14%)
+++					1 (14%)	1 (14%)	
++++					4 (57%)		
Positive Cases (n.%)		**4 (57%)**	**5 (71%)**	**2 (29%)**	**7 (100%)**	**7 (100%)**	**5 (71%)**
Invasive	**5**						
-		5 (100%)	1 (20%)	3 (60%)	0 (0%)	2 (40%)	3 (60%)
+			4 (80%)	1 (20%)	2 (40%)	2 (40%)	1 (20%)
++				1 (20%)		1 (20%)	1 (20%)
+++					3 (60%)		
++++							
Positive Cases (n.%)		**0 %**	**4 (80%)**	**2 (40%)**	**5 (100%)**	**3 (60 %)**	**2 (40%)**
Total positive cases (NMIBC+MIBC)	**29**	**6 (21%)**	**13 (45%)**	**8 (28%)**	**29 (100%)**	**26 (86%)**	**14 (48%)**

Score of extension Staining	
Negative	-
>0-15%	+
16-30%	++
31-45%	+++
≥46%	++++

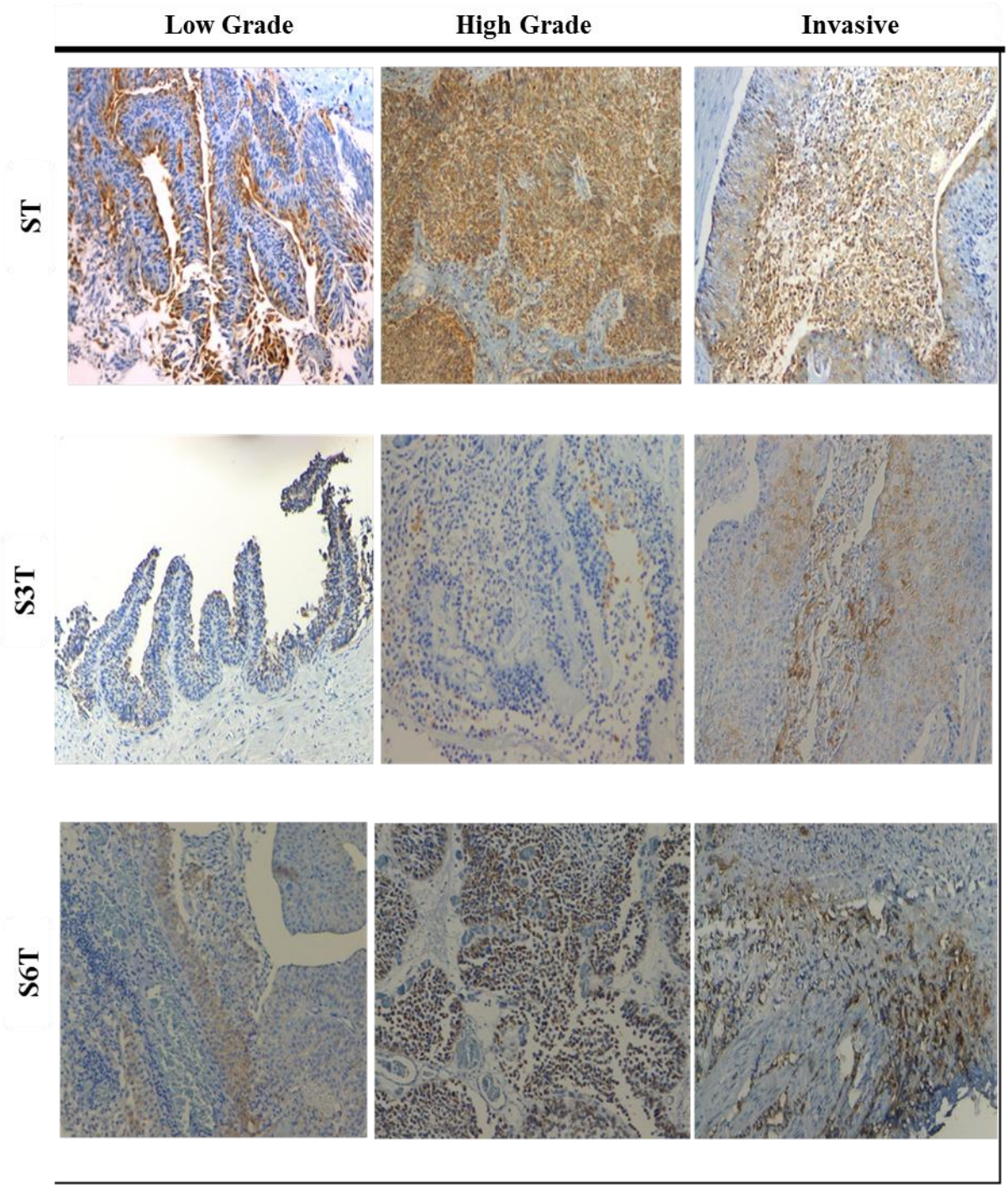


Figure 1*:* Illustrative representation of immunohistochemistry of (ST, S3T and S6T) for each of the types of lesions presents in this study (Low-Grade, High-Grade and Invader).

3.2. Expression of S6T and S3T

The enzymatic treatment of the tissues with a *β*-(1-3)-galactosidase allowed positive staining in, up until now and non-reactive sections for anti-STn monoclonal antibody TKH2 (Figure 2 A/C). This removed *O*-3 linked Gal residues from ST exposing a STn antigen and demonstrating, for the first time, that bladder tumors express S6T. This antigen was found in 86% of the tumors, predominantly among high grade NMBIC (100%). Approximately 89 % and 60% of low grade and MIBC correspondently, were also positive. S6T was further evaluated in FFPE healthy urothelium from six necropsied male individuals, available in the laboratory at the time of the experience. The absence of antigens in these tissues allowed concluding that S6T is a bladder tumor specific glycosylation. It further suggests that ST6GalNAC-I and/or II or ST6GalNAC-IV known to be involved in the *O*-6 sialylation of Tn antigens, may be over-expressed in bladder tumors. However, other factors such a reduced expression of the glycosyltransferases responsible by chain elongation and alterations in the secretory pathways may also contribute for it. Given the clinical relevance of this observation further studies should be conducted to evaluate its biomarker value. Taking into account the pancarcinoma nature of simple mucin-type *O*-glycans this strategy can be applied to screen other solid tumors for this particular glycan.

On the other hand, incubation with a α-neuraminase specific for *O*-3 linked residues allowed positive staining in, up until now, non-reactive sections for anti-T mononclonal antibody 3C9 (Figure 2 B/D). This enzyme removed *O*-3 linked Neu5Ac residues from S3T, thus exposing a T antigen. According to a previous study by Videira *et al.*,(2009) [13], bladder tumors over-express ST3Gal-I, which is the sialyltransferase responsible by the *O*-3 sialylation of the T antigen. These results now provide the structural confirmation that S3T is present in bladder tumors. However, S3T has a more restricted pattern of expression than S6T, as it was only found in 48% of the cases. It was more pronounced among high grade tumors (71% of the cases) when compared to low grade and MIBC (41% and 40 % respectively). The lack of healthy urothelium at the time of the experiment did not allow determining whether this antigen may be present in non-pathological conditions. It was also observed that all S3T positive cases were also S6T positive, which demonstrates that both sialylated forms of the T antigen can coexist within

the same tumor. However, 34% of the cases (n=10), predominantly low grade (70% low grade, 10% high grade, 20% MIBC) were S6T positive but did not express S3T. This suggests either an over-expression of *O*-6 sialyltransferases acting on the T antigen (ST6GalNAC-I/II/IV) or the lower expression of *O*-3 linked sialyltransferases (ST3Gal-I/II/V). Competitive factors between both enzymes as well as environmental changes may also play an important role in the predominance of S6T over S3T.

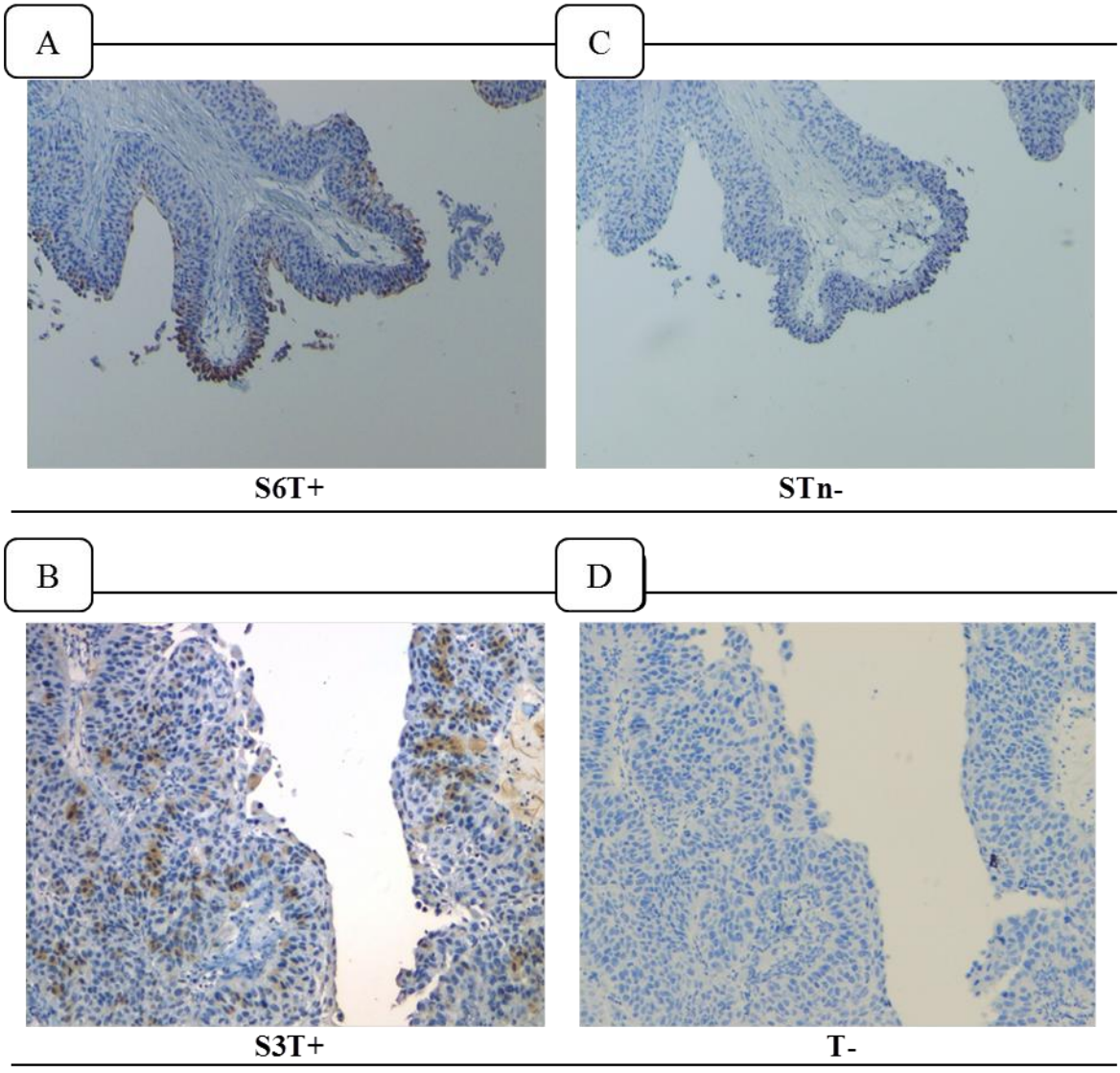
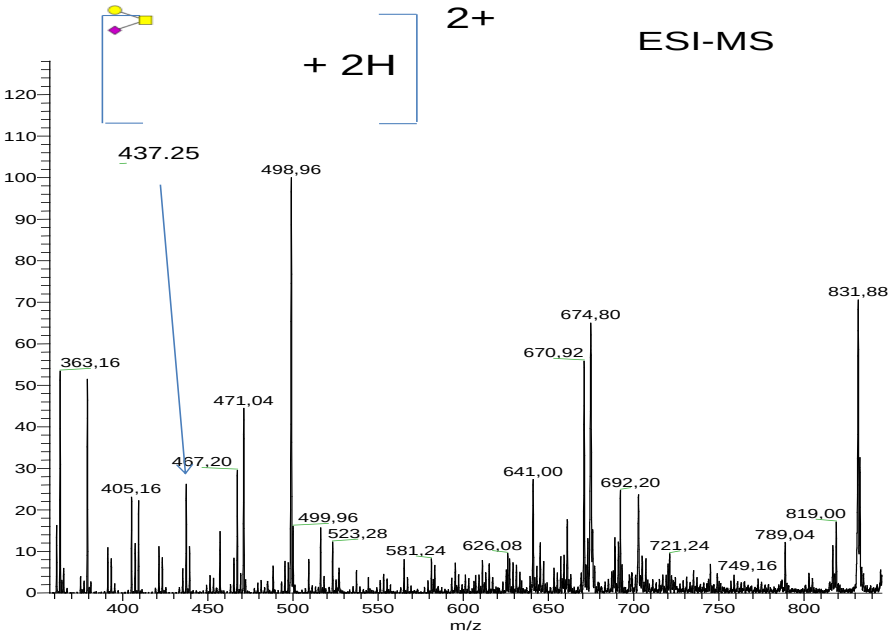


Figure 2: Positive staining of S6T (A) and S3T (B) and non-reactive sections of anti-STn (C) and anti-T (D).

Given the novelty of these observations, S6T and S3T were also accessed by mass spectrometry. Proteins were first isolated from FFPE over-expressing these antigens, chemically de-*O*-glycosylated as described in the Materials and Methods section and analyzed by nano-LC-ESI-MS/MS. However, our preliminary studies using SLe^x, that exhibits an *O*-3 linked Neu5Ac residue, have demonstrated that sialic acids can be lost under soft ionization methods such as electrospray. To overcome this limitation by reducing the lability of acidic moieties, the glycans were permethylated prior to analysis. This also promoted and equalization of their chemical properties allowing the detection of both neutral and acidic species in positive ion mode. This approach allowed the identification of an ion at *m/z* 437.25 corresponding to $[ST+2H]^{2+}$. This assignment was confirmed based on the characteristic product ion spectra presented in Figure 3. This MS/MS spectra predominantly exhibited ions resulting from combination of X-type cross-ring fragmentations with one or several B-, C-, Y- and Z-type fragmentations (nomenclature fragmentation according to Domon and Castello (1988) [25] that have been summarized in Table 2 and identified in Figure 3. Combined cross-ring and glycosidic bond cleavages occurring at both the reduced and non-reduced terminal ends are characteristic of permethylated glycans under tandem conditions [26]. Among these, are the ions at *m/z* 291.15 and 309.03 corresponding to a sialic acid residue linked to a Gal residue (Figure 3; Table 2) characteristic of S3T. On the other hand, the product ions at *m/z* 364.27 and 406.34 resulting from sialic acids linked to a GalNAc residue are characteristic of S6T. Other, less intense ions characteristic of both isomers are summarized in Table 2. These reporter ions demonstrate the coexistence of the two isomeric structures in samples over-expressing both S3T and S6T, thus validating immunohistochemistry observations.

A)



B)

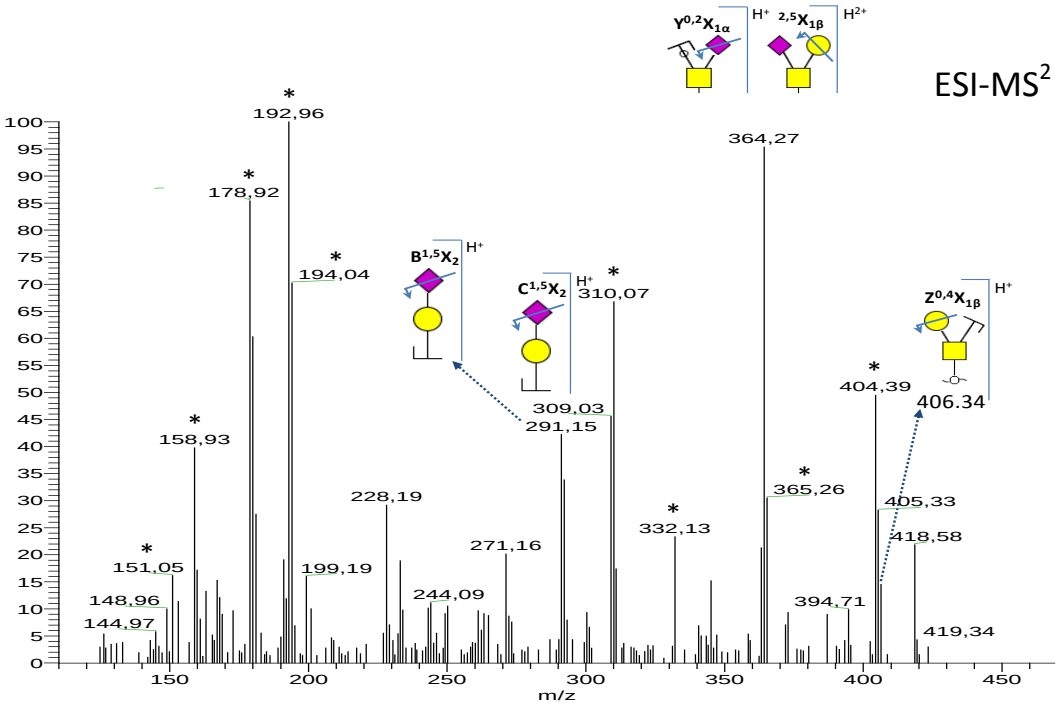
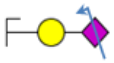
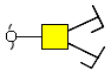
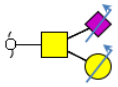
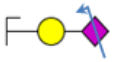


Figure 3: **A)** ESI-MS spectrum showing the ion at m/z 437,25 assigned to $[ST+2H+]^{2+}$. **B)** Partial overview of the product ion spectra of the ion at *m/z* 437.25 assigned to $[ST+2H]^{2+}$. The more intense reporter ions for S6T at *m/z* 364.27 and 406.34 and the reporter ions for S3T at *m/z* 291.15 and 309.3 have been highlighted. The fragmentations are presented according to the nomenclature introduced by Domon and Costello [37]. "*" signals resulting from the combination of cross-ring and glycosidic bound fragmentations occurring at both the reducing and non-reducing ends. (○ - Gal; □ - GalNAc; ◆ - Neu5Ac).

Table 2: Resume of the less intense ions observed in the spectrum of Figure3 that are reporters of S3T and S6T. (○ - Gal; □ - GalNAc; ◆ - Neu5Ac).

ion (*m/z*)	Charge	Ion type	Assigned structure	*O*-glycan
167.10	2H	$B^{0,3}X_2$		S3T
228.19	2H	$C^{1,4}X_2$		S3T
244.08	H	ZZ		S6T
261.19	$2H^+$	$^{1,5}X_{1\beta}$ $^{0,4}X_{1\alpha}$		S6T
418.58	H	$B^{0,4}X_2$		S3T

3.3. Expression of STn

Despite its pancarcinoma nature and association with malignancy, the expression of STn in bladder cancer remains uncharacterized. This study is now showing that STn is mostly expressed in high grade and invasive tumors (71 and 80% respectively), whereas only 24% of low grade cases were positive. The majority of the positive cases presented a low extension of expression (<15%), of focal and polydisperse nature throughout the tumor. STn was mostly found in cells of the basal layer (Figure 4 A); yet in tumor areas presenting extensive staining (>50%) (Figure 4 B), it could also be detectable in papillary urothelium and invasive fronts (Figure 4 C). Moreover, we have found that whenever present in the tumor, STn was also detectable in the adjacent but not in the distal mucosa. Hence, cells neighbouring the tumor are thought to carry significant alterations that result in the expression of this antigen. Further studies should be conducted to access the

biomarker value of STn in the delimitation of tumor boundaries. Adding to these observations, STn was not found in healthy urothelium from necropsied male individuals (n=6), thus demonstrating its tumor-specific nature. These results further suggest that ST6GalNAC-I or II may be over-expressed in high grade and MIBC.

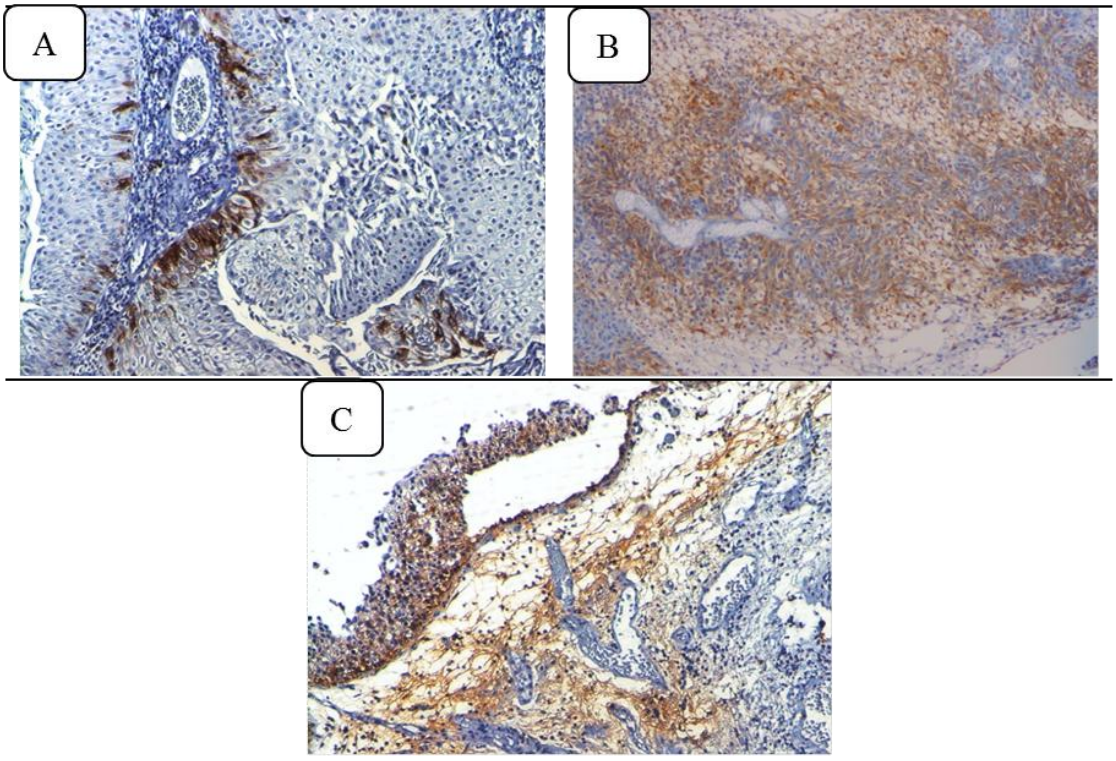


Figura 4: Representation of the STn positive staining. (A: Basal layer staining; B: Extensive STn staining (> 50%); C: Papillary and Invasive fronts staining)

4. Conclusions

The evaluation of the expression of *O*-glycosylated epitopes in 29 neoplastic lesions of the bladder, performed by immunohistochemistry, revealed a high incidence of expression of sialylated antigens in bladder tumors, irrespectively of their classification. Moreover, two novel sialylated species were detected in these tumors, S6T and S3T, however with distinct patterns of positivity. S6T showed broader expression (~90% of the tumors) and was particularly prevalent among high grade cases. Conversely, S3T expression was more restricted (48% of the tumors) being that, it was also associated with high-grade tumors. Furthermore, it was concluded that S6T prevailed over S3T, particularly among low grade tumors and that S6T was a tumor-specific type of glycosylation. It was also observed that the STn antigen expression was almost restricted to more advanced bladder tumors, namely high grade and MIBC. This observation is of particular interest since high-grade tumors entails an elevated risk of recurrence and progression to muscle invasive disease. The identification of high-grade associated biomarker may aid tumor classification, provide therapeutic guidance and novel targets for immunotherapy approaches.

Having established a pattern of expression for simple mucin type *O*-GalNAc glycans in bladder cancer further studies should be conducted to access the clinical value of these observations.

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Chapter V

Tumor associated carbohydrate antigen Sialyl-Tn as response predictor of Bacillus Calmette-Guérin immunotherapy in High-Risk Bladder cancer patients

Abstract

The most effective treatment for bladder cancer patients exhibiting a high-risk of recurrence/progression is transurothelial resection followed by immunotherapy with Bacillus Calmette-Guérin (BCG). Even though this therapeutics successfully delays recurrence, approximately 15% of the initial responders end up progressing to muscle invasive disease. Foreseeing those best served by an alternative or even more aggressive such as early cystectomy, would avoid disease progression, reduce disease burden and decrease health expenses. However, at the moment there are no biomarkers to predict BCG therapeutics outcome.

In chapter IV it was demonstrated that that high-grade tumors, constituting the majority of the cases indicated for BCG immunotherapy, abnormally express the carbohydrate antigen STn. Another study has shown that the expression of STn by bladder cancer cell lines resulted in higher adhesion and internalization of the bacillus. Since these events that are associated with an increased probability of response, it is hypothesized that glycosylation may dictate the success of therapeutics. Thus, this study was dedicated to determining the predictive value of STn in BCG immunotherapy outcome. STn was evaluated by immunohistochemistry in a retrospective series of 94 high-risk tumors. This showed that positivity for this antigen was associated with a better response to treatment (p=0.024). Moreover it highlighted a trend towards an increased survival. The incorporation of the S6T antigen, which has a STn domain, into this model increased the overall response (p=0.001) and recurrence free survival (p=0.001). Further studies should be conducted to fully clarify the implications of these observations in the management of bladder tumors.

Key words: Non-muscle-invasive bladder cancer**,** bacillus Calmette-Guérin, STn, S6T, high-risk bladder tumors.

1. Introduction

To reduce the risk of relapse in high-risk patients, comprehending high-grade papillary tumors but also carcinoma *in situ*, and multifocal and recurrent lesions, are submitted to intravesical instillations with Bacillus Calmette-Guérin (BCG) after transurothelial resection of the tumor [1, 2]. Although the management of these patients has significantly improved, BCG treatment one third either do not respond or show intolerance to BCG, 70% of the responders relapse and approximately 15% progress to muscle invasive tumors [3, 4]. Upon therapeutic failure and/or muscle invasion the patient is faced with cystectomy [5]. Foreseeing those best served by an alternative treatment or early cystectomy, would avoid progression, reduce disease charge and decrease health expenses. However, at present, the risk of relapse and progression is based exclusively with several clinical and histopathological factors (number and size of tumors, number of previous relapses, local staging (T) and degree of differentiation) [6, 7]. So, despite some promising targets, there are no established biomarkers to determine the success of BCG immunotherapeutics [8,13].

The success of BCG immunotherapy resides mostly on the capability of the bacillus to recognize and efficiently bind to tumor cells. The bacillus is then internalized triggering tumour cell apoptosis and host adaptive immune responses [4]. Several lectins able to bind human cell-membrane glycans have been identified in *Mycobacterium spp*., including BCG [1], demonstrating that glycosylation acts as mediator of adhesion. In agreement with these observations, it was also noted that BCG has a higher rate of adherence and internalization to cells expressing STn [9]. Moreover, in chapter IV it was demonstrated that high-grade tumors, entailing a high-risk of recurrence with progression, abnormally express the carbohydrate antigen STn. This suggests that tumor-specific glycosylation could dictate the course of BCG therapeutics. Thus, the present work is dedicated to evaluating the role of STn as response predictor to BCG immunotherapy in high-risk tumors.

2. Material and Methods

2.1. Population

In this study was used a retrospective series of 94 cases with high risk NMIBC. The patients were submitted to therapy with BCG on the Portuguese Oncology Institute of Porto, between 1998 and 2006. All patients had received intravesical instillation of BCG for 6 consecutive weeks, starting 2-3 weeks after surgery (iBCG) and 56.4% were submitted to mBCG schedule (iBCG + maintenance protocol with instillations every 2 or 3 months during 2 years).

The male:female sex ratio was of 78:16. The patients were followed every 3 months for the first year, every 6 months for the second year and every 12 months thereafter by cystoscopy and urine cytology. The tumor recurrence was defined as the appearance of a newly found bladder tumor after the treatment, with at least one tumor-free cystoscopy between. So, the non-responders were defined as patients submitted to BCG treatment with tumor recurrence.

Finally, recurrence-free survival (RFS) was defined as the period between the beginning of BCG treatment until the date of the most recent cystoscopy or recurrence date. All clinical and pathological information was obtained from medical records being that the institutional ethics committee approved the study.

2.2. Evaluation of STn and S6T by immunohistochemistry

STn was evaluated by immunohistochemistry in formalin fixed paraffin embedded tissues using monoclonal antibody TKH2 as described in chapter IV. S6T was also evaluated in tumors showing negative staining for TKH2.

2.3. Statistical analysis

The statistical analysis of data was performed using the computer software package Statistical for Social Sciences, SPSS for Windows (version 15.0). Chi-square analysis was used to compare categorical variables. Logistic regression was used to predict the outcome of BCG immunotherapy based on STn and S6T expression and to adjusted variables that could affect the outcome. The odds ratio (OR) and its 95% confidence interval (CI) were calculated as a measurement of the association between the expression of *O*-glycosylated structures and recurrence. Further, multiple Cox regression analysis was used to assess the effect of individual epitopes on the time to recurrence in BCG-treated patients and to adjusted variables that could affect the outcome.

3. Results and Discussion

The first part of this study was dedicated to determining the influence of several clinicopathological features (stage, grade, size, number of tumors, presence of CIS, recurrence status), treatment scheme (iBCG, mBCG) and other variables (sex) in BCG treatment outcome (responders *vs.* non-responders) (Table 1). Using a Using Chi-square analysis, the only variable correlating with recurrence is the treatment scheme (iBCG and mBCG). It was observed that the mBCG scheme (iBCG + maintenance protocol with instillations every 2 or 3 months during 2 or 3 years), was more effective (Table 1). These results are in agreement with previous studies that states that maintenance BCG is required in order to achieve the best therapeutic results [10]. Thus, subsequent studies addressing the estimation of STn expression in the outcome have been carried out taking in consideration this effect.

Table 1: Relation between patients, clinical and tumor characteristics and BCG treatment outcome.

Variables		**Total**	**Responders**	**Non-Responders**	**p**
		n (%)	n (%)	n (%)	
Sex					
	Male	78 (83.0%)	47 (60.0%)	31 (40.0%)	0.585
	Female	16 (17.0%)	11 (68.8%)	15 (31.2%)	
Stage					
	Ta	40 (42.6%)	23 (57.5%)	17 (42.5%)	0.524
	T1	54 (57.4%)	35 (64.8%)	19 (35.2%)	
Grade					
	Low	21 (22.3%)	13 (61.9%)	8 (38.1%)	1
	High	73 (77.7%)	45 (61.6%)	28 (38.4%)	
Size (cm)					
	<3	62 (66.0%)	38 (61.3%)	24 (38.7%)	0.823
	≥3	31(34.0%)	20 (64.5%)	11 (35.5%)	
Tumor number					
	Unifocal	51 (54.3%)	28 (55.0%)	23 (45.0%)	0.201
	Multifocal	43 (45.7%)	30 (70.0%)	13 (30.0%)	
CIS					
	No	88 (93.6%)	54 (61.4%)	34 (38.6%)	1
	Yes	6 (6.4%)	4 (67%)	2 (32.3%)	
Recurrence Status					
	Primary	48 (51.0%)	31 (64.6%)	17 (35.4%)	0,672
	Recurrent	46 (49%)	27 (58.7%)	19 (41.3%)	
BCG schedule					
	iBCG	41 (43.6%)	20 (48.8%)	21 (51.2%)	0.032
	mBCG	53 (56.4%)	38 (71.7%)	15 (28.3%)	

The Table 2 shows the correlation between the expressions of STn with the response to treatment. In this context, 69.4% of cases that responded to treatment with BCG expressed STn. With respect to non-responders, the expression of STn was seen in 30.6% of cases. Thus, the frequency of STn expression is higher in patients that responded to BCG, when compared with non-responders group (p = 0.024). Moreover, the risk of developing disease was approximately 65% lower in patients expressing STn (OR = 0.346).

However, taking into account the existence of a high percentage of STn negative cases was incorporated into the study, the evaluation of the expression of a sialylated structure very similar to the STn. In an attempt to improve this predictive model S6T, which may be considered an STn-like antigen, has been evaluated in STn negative cases. This permitted to improve both the correlation with response (p = 0.001) and lowered the risk of developing disease (OR = 0.170). Such, occurs due to a reclassification of STn negative cases responding to BCG to (STn/S6T) positive (Figure 1).

Table 2: Distribution expression of STn and S6T and risk of recurrence after BCG therapy.

	BCG response				
	Responders	**Non-Responders**	OR[a]	**95%CI**	P*
STn					
Negative	15 (46.9%)	17 (53.1%)	1,0	Referent	
Positive	43 (69.4%)	19 (30.6%)	0,346	0,132-0,871	0.024
S6T+STn					
Negative	8 (33.3%)	16 (66.7%)	1,0	Referent	
Positive	50 (71.4%)	20 (28.6%)	0,170	0,059-0,489	0.001

[*] logistic regression

[a] adjusted for treatment sheme (iBCG/mBCG)

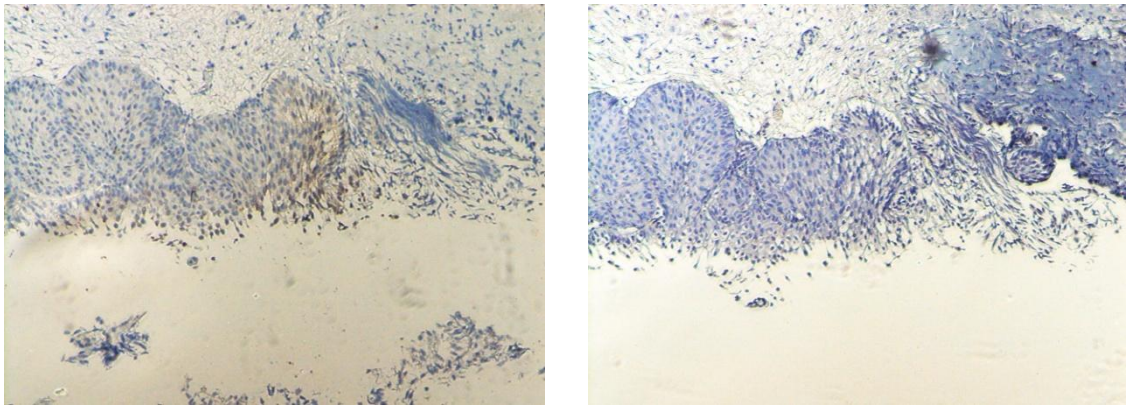

Figure 1: STn negative and S6T positive case that responded to BCG immunotherapy.

Finally, the Cox regression analysis adjusted to treatment scheme was performed to assess the individual effect of STn and STn+S6T on the time to recurrence in BCG-treated patients. Table 3 shows that patients with STn positive tumors have a trend to a lower risk of early recurrence after BCG treatment (HR=0,551; 95%CI: [0.284-1.070]). Likewise, it was observed that the cases involving positive STn+S6T show a statically significant lower risk of early relapse after the application of therapy in question (HR = 0.300, 95% CI [0.151 to 0.596]). This reinforces the idea that the expression of STn-like *O*-glycosylated structures (STn and S6T) contribute to a decreased risk of developing the disease in question after treatment with BCG. So, the immunotherapy with this bacillus may be effective in these cases. These observations reinforce previous studies suggesting an active role of STn in BCG immunotherapy by promoting adhesion an internalization of the bacillus. Whether the described association results from a direct recognition and binding of the bacillus to tumor cells remains unknown. The abnormal nature of STn and STn-like glycans may also play a determinant role in a better response to BCG. These glycans have been proven immunogenic as demonstrated by their exploitation in vaccine formulations [11]. Yet, the immunosuppressive environment of tumors often prevents the development of an effective response [12]. However, several reports have demonstrated that the use of immunological boosters may overcome this limitation [12]. It may be the case that BCG is acting in the same way, thus promoting the elimination of tumor cells expressing altered

glycosylations. Further studies should be conducted to access this matter and may render important clues about novel immunotherapeutics against bladder cancer using altered glycosylation.

Table 3: Multivariated analysis and risk estimation of sTn and S6T influence

	HR	**95%CI**	**P**
STn			
Negative	1.0	Referent	
Positive	0,551	0,284-1.070	0.078
S6T+STn			
Negative	1.0	Referent	
Positive	0,300	0,151-0.596	0.001

4. Conclusion

One of the most critical aspects in managing bladder cancer in patients with a high-risk of recurrence/progression is to predict the outcome of adjuvant immunotherapy with BCG. Moreover, at the moment there are no biomarkers to anticipate this event.

Herein it has been demonstrated that the expression of abnormal *O*-glycans STn and S6T correlated with a better response to this treatment. It is expected that these glycans in association with other BCG-response associated molecules already identified [8] may allow the establishment of a predictive panel that can guide therapeutic decision.

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Chapter VI

General Conclusions

1. General Conclusions

The first part of this work has been dedicated to defining a pattern of expression for simple mucin type *O*-GalNAc glycans in bladder cancer. In agreement with observations for other solid tumors, it was concluded that sialylated forms predominate over neutral glycans. Also, two novel sialylated species were detected in these tumors, S6T and S3T. S6T prevailed over S3T, particularly among low grade tumors and was found to be a tumor-specific type of glycosylation. Conversely, S3T was more frequently expressed in high-grade tumors. Contrasting with the monosialylated forms of the T antigen, STn expression was almost restricted to more advanced bladder tumors, namely high grade and MIBC. This observation is of particular interest since high-grade tumors entails an elevated risk of recurrence and progression to muscle invasive disease. The tumor-specific nature of these antigens and their differentiated pattern of expression make believe they could be valuable biomarkers and constitute targets for novel therapeutics.

Finally, it was evidenced that the expression of abnormal *O*-glycans STn and S6T is correlated with a better response to treatment with BCG. It is expected that these glycans in association with other BCG-response associated molecules already identified may allow the establishment of a predictive panel that can guide therapeutic decision.